REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	18 March 1996	Final 9/1/92 - 10/31/95

4. TITLE AND SUBTITLE

Gene Regulation in Memory Formation and Circadian Rhythm

5. FUNDING NUMBERS

F49620-92-J-0494

2312/BS/CS

6. AUTHOR(S)

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AFOSR-TR-96
0139

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Air Force Office of Scientific Research
Bolling AFB, DC 20332-6448

NL

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Funding Agency Responsible Individual: Dr. Genevieve Haddad
Phone: (202) 767-5023

12a. DISTRIBUTION/AVAILABILITY STATEMENT

DISTRIBUTION STATEMENT A
Approved for public release
Distribution Unlimited

12b. DISTRIBUTION CODE

19960408 076

13. ABSTRACT (Maximum 200 words)

Although it has been clear for some time that regulation of gene expression is a key process in the development of organisms, the degree to which regulation of gene expression plays a role in the life of adults has been less clear. The general objectives of this research were to investigate the role of gene expression in circadian rhythms and in memory formation. The first stage of this research required obtaining clones of genes for proteins thought to be involved in learning and memory and circadian rhythms. We cloned the Aplysia genes for porin, protein 3, phosphoglycerate kinase, calmodulin, BiP, and HSP70. Using riboprobes made from these gene sequences, we developed sensitive techniques to measure levels of mRNA in eyes and in a cluster of a few hundred sensory neurons. Using these techniques, we discovered that the expression of most of these genes was regulated by light and/or 5-HT, treatments known to perturb the clock and to induce the formation of memory. We have now begun to study regulation of this expression in greater detail. Overall, these studies on gene regulation have further implicated some of these genes/proteins in mechanisms of the circadian system and the system by which memories are induced and maintained. A particular outstanding result was our discovery that a developmental gene, known in Drosophila as Tolloid, appeared to be involved in memory.

14. SUBJECT TERMS	15. NUMBER OF PAGES
mRNA levels, Aplysia	
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	UL

NSN 7540-01-280-5500

Title Page

Report of grant number F49620-92-J-0494

GENE REGULATION IN MEMORY FORMATION AND CIRCADIAN RHYTHMS

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Final Technical Report for period 1 September 1992 - 31 October 1996

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I. Introduction and Summary

Although it has been clear for some time that regulation of gene expression is a key process in the development of organisms, the degree to which regulation of gene expression plays a role in the life of adults has been less clear. Now, it is evident that gene expression has two important roles which have major impacts on the behavior and physiology of adults. One role involves gene expression in the circadian system, and the other involves gene expression in learning and memory. Because *Aplysia* is an excellent organism for studies of learning and memory and circadian rhythms, and similar techniques may be used to study both areas, we have been investigating the role of gene regulation in circadian timing in the isolated eye and in mechanisms responsible for the formation of long-term memories in the rest of the nervous system. The information gained from these experiments should help us understand similar issues in higher animals and, perhaps, man as well.

The invertebrates, in general, and *Aplysia*, in particular, recently have aided our understanding of a number of neurobiological mechanisms in higher organisms. For example, very recent findings on the molecular basis of learning and memory in *Aplysia* have been directly applied to learning in mammalian brains. One such example is the importance of transcription and translation in long-term memory, first demonstrated in *Aplysia* and now being demonstrated in research on LTP in the hippocampus (Montarolo, *et al.*, 1986; Huang, *et al.*, 1995). A second example is the importance of CREB, a transcription factor activated by cAMP, in the induction of long-term memory, first established in *Aplysia* and recently demonstrated to be involved in *Drosophila* and the mouse (Dash, *et al.*, 1990; Stevens, 1994). Furthermore, in circadian rhythms, our initial observations of phase shifting by serotonin, cAMP, and cGMP have been shown to have exact parallels in the effects of 5-HT, cAMP, and cGMP in the vertebrate SCN (Koumenis and Eskin, 1992; Prosser, *et al.*, 1989; Medanic and Gillette, 1992).

Our original experimental studies consisted of screening for proteins that might play roles in circadian timing or in learning and memory. We "screened" for proteins whose synthesis was regulated by entraining agents (light, serotonin) and for proteins whose synthesis was regulated by treatments that induced memory (electrical nerve stimulation, serotonin) (Koumenis, *et al.*, 1995). Using 2D-gel electrophoresis to find proteins and then protein microsequencing and cDNA cloning techniques to identify them, we focused on 3 proteins for study in the circadian rhythm projects (porin, BiP, HSP70, 34K, 27K) and 3 proteins for study in the learning and memory projects (calmodulin, phosphoglycerate kinase, and protein 3).

One of the major objectives of the Air Force grant was to study the regulation of the genes for these proteins. To do this, we first had to develop techniques in my laboratory for cloning *Aplysia* cDNAs for the "rhythm" and the "learning" proteins. This has been accomplished very well as we have now cloned more than a dozen *Aplysia* cDNAs and we have developed a very sensitive method for measuring levels of mRNAs, the ribonuclease protection assay (RPA). Using RPAs, we can measure levels of mRNAs in very small amounts of tissues (isolated eyes and clusters of sensory neurons

containing approximately 200 cells). With the development of these techniques, we studied the regulation of the genes for the "rhythm" proteins and the "learning" proteins.

In the circadian rhythm project, we found that levels of mRNAs for some of the putative eye clock proteins (porin, BiP) were regulated by light and 5-HT (Sloan, *et al.*, 1995). This represents one of the first such studies in which mRNAs were shown to be regulated by entraining agents. A truly elegant paper by Crosthwaite, Loros, and Dunlap on light regulation of FRQ in *Neurospora* represents the first demonstration that a clock component is regulated by light (Crosthwaite, *et al.*, 1995). We recently have initiated studies to investigate whether the mRNAs regulated by light and 5-HT exhibit circadian rhythms. Also, we initiated studies of rhythms of mRNA in tissues other than the isolated eye. We have obtained preliminary data showing that several mRNAs (e.g., C/EBP) exhibit rhythms in the isolated abdominal ganglion or eyes of *Aplysia* (Hattar and Eskin, 1996).

In the learning and memory project, we found that 5-HT regulated some "learning" genes (PGK, calmodulin, protein 3), and we began investigating whether similar changes occurred in intact animals during behavioral training (Eskin, *et al.*, 1993). Also, considerable effort was devoted to discovering the identify of protein 3 (MacPhee, *et al.*, 1995). We also investigated proteins that were affected by an associative training protocol (Noel, *et al.*, 1994). We found one such protein, obtained a partial amino acid sequence of it, then cloned and sequenced a cDNA for the protein. Finally, we used a relatively new technique, differential display reverse transcription - PCR (ddRT-PCR), to look for mRNAs whose levels were altered by treatments with 5-HT. This technique is conceptually similar to the 2D-gel technique for proteins in that it allows one to investigate changes in levels of specific, but unknown, mRNAs. However, the ddRT-PCR technique is a much more sensitive method than the 2D-gel technique. Once an mRNA whose level is changed by some treatment is found with ddRT-PCR, one must confirm the change using another technique to measure mRNAs, e.g., RPAs. Using ddRT-PCR, we found an mRNA whose level was significantly increased by 5-HT treatments and behavioral training (Liu, *et al.*, 1995). The *Aplysia* gene coding for this mRNA has been sequenced completely. This *Aplysia* gene belongs to a family of developmentally-regulated genes including the gene that codes for human bone morphogenetic protein 1 and *Drosophila tolloid*. This is an extremely exciting and potentially very important finding because this gene may play a role in regulating, and then stabilizing morphological changes of synapses, a process that is known to accompany learning in both vertebrates and invertebrates. Future studies of *Aplysia tolloid* place us in a position where we can investigate specific issues dealing with mechanisms for the maintenance of memory.

Another objective of the Air Force proposal, which was common to both learning and memory and circadian rhythms, was the development of techniques to interfere with gene expression. Some promising preliminary data were obtained as we demonstrated that the heat shock response of HSP70 in abdominal ganglia could be blocked by exposing ganglia to phosphorothioate nucleotides containing the heat shock response element.

The last objective of the Air Force project was to investigate whether *Halobacteria* expressed circadian rhythms. The recent discovery that a prokaryote, cyanobacteria, contains a circadian clock

raised the possibility that other prokaryotes might also have clocks available to study. We chose to investigate *Halobacteria* because it was in a different kingdom from cyanobacteria, and it potentially has advantages, e.g., it is not photosynthetic. A graduate student, Mark Sloan, with the help of Dr. M. Benedik, spent about one year studying *Halobacteria*. To date, we have been unable to find proteins whose synthesis is regulated by light and we have been unable to find any evidence for a circadian clock in cyanobacteria.

Under the sponsorship of the Air Force Grant, a number of other preliminary studies were initiated. In one of these projects, we found that the circadian rhythm in eyes undergoes striking age-related changes (Sloan and Eskin, 1996). In another of these projects, we found that glutamate uptake was regulated by serotonin. This regulation of uptake may represent another mechanism for regulation of synaptic plasticity that could underlie some memories. The initial findings in these two areas indicate that these two projects warrant further study.

II. Specific Progress

A. Regulation of genes for proteins that appear to be involved in learning and memory

The major objective of this research is to provide insights into the regulation of biochemical and molecular events that occur while organisms learn and form memories. We are examining the hypothesis that regulation of gene transcription and protein translation is involved in the formation of long-term memories. Moreover, a cascade of transcription events is hypothesized to affect the translation of a diverse array of proteins that control a broad spectrum of cellular functions. The specific framework of our studies is sensitization of the defensive tail–siphon withdrawal reflex in *Aplysia*, and mRNA and protein changes in large homogeneous clusters of bilaterally-symmetrical sensory neurons in the pleural ganglia. These sensory neurons are key components of the reflex and its plasticity. We began this series of experiments by asking "Does 5–HT change the synthesis of any specific mRNAs?" Initially, we used methods of *in vitro* translation to search for mRNAs. More recently, we have used differential display of reverse transcription - PCR (ddRT-PCR) to search for mRNAs affected by 5-HT.

1. Serotonin affects four mRNAs in pleural–pedal ganglia

To determine whether 5–HT produces changes in levels of mRNAs, we used *in vitro* translation of cellular mRNAs along with 2D–PAGE of the protein products (Eskin, 1993). Isolated experimental pleural–pedal ganglia were exposed to 5-HT for 1.5h. At the end of the 5–HT treatment, the experimental and matched control ganglia were frozen, RNA was extracted, and then translated *in vitro* with rabbit reticulocyte lysate in the presence of ^{35}S–methionine. The labeled proteins were then separated by 2D–PAGE and analyzed by fluorography and computerized densitometry. In this way, the OD of a given protein on the film is proportional to the amount or "translatability" of the mRNAs in the extract from the ganglia. Serotonin significantly affected 4 mRNAs. The incorporation

of label was consistently increased into 3 proteins and decreased into 1 protein when RNA from 5-HT treated ganglia was translated, as compared to RNA from untreated ganglia.

2. Identification of the mRNAs affected by 5–HT

(a) The mRNA of Protein 1 codes for calmodulin. One of the 4 mRNAs affected by 5-HT coded for a protein (protein 1) whose M_r and pI (17KD and 3.9) was similar to that of calmodulin (CaM). To determine if the *in vitro* translated protein was produced from a CaM mRNA, CaM mRNAs were selectively removed from pleural–pedal RNA by hybridization with an oligo complementary to CaM followed by digestion with RNase H before the *in vitro* translation procedure. Incorporation only into the 17KD, pI 3.9 protein, was reduced by this procedure demonstrating that the mRNA of protein 1 codes for CaM. Furthermore, anti–rat CaM antibody specifically recognized the 17KD, pI 3.9 protein in a Western blot of a 2D–gel with protein from pleural–pedal ganglia. **(b)** The mRNA of Protein 2 codes for phosphoglycerate kinase. Microsequencing techniques were used to obtain partial sequences of protein 2. A sequence of 45 amino acids obtained from protein 2 was found to be 88% identical to a glycolytic enzyme, human 3–phosphoglycerate kinase II (PGK II). To confirm that the protein we studied is PGK, we investigated if antibodies made against human PGK would recognize the *Aplysia* protein on Western Blots of 2D-gels. The antibody reacted with the protein we had identified through micro-sequencing as PGK. Recently, we used a human cDNA clone of PGK to screen a Lambda Zap cDNA library made from *Aplysia* head ganglia (obtained as a gift from Drs. Nagle, Knock, and Kurosky, Univ. of Texas Med. Branch, Galveston, TX). A positive clone has yielded 645 base pairs of the coding region that is around 85% identical to human phosphoglycerate kinase. Therefore, a cDNA coding for PGK has been cloned. **(c)** The mRNA of Protein 3 codes for a novel protein. Sequences of 27 and 30 amino acids were obtained from 2 peptides derived from the ganglion protein corresponding to protein 3. Subsequently, oligonucleotides were made, based on portions of the amino acid sequences of each peptide. These oligonucleotides were then used in the polymerase chain reaction (PCR) to amplify, from *Aplysia* nervous system cDNA, the intervening region between the 2 peptides. The PCR product contained sequence between the 2 peptides which coded for an additional 10 amino acids, giving us a total of 67 amino acids for protein 3. Database searches revealed no protein with an amino acid sequence similar to this partial sequence of protein 3. To explore further the identity of protein 3, the PCR product generated from the 2 peptides of protein 3 was used to screen the cDNA library made from *Aplysia* head ganglia. A positive cDNA clone of 1750 base pairs was identified and has been sequenced. The deduced amino acid sequence of the clone yielded a 'novel' protein of 53 Kd MW and 4.85 pI. Protein 3 may be multifunctional: the N-terminal region of Protein 3 is similar to reductases, whereas the Glu-rich C-terminal region of Protein 3 is similar to an immediate early protein. Isolation of the cDNA for this protein has set the stage for investigation of its cellular function as well as its role in long-term facilitation and in behavioral sensitization. **(d)** Protein 4 has not been identified because we could not find the *in vitro* translated protein in 2D-gels of ganglia

that were labeled while intact. Presumably, this lack of correspondence is due to protein 4 being processed in endoplasmic reticulam and golgi which are not included in the *in vitro* translation lysate.

3. Confirmation that 5-HT affects levels of mRNAs of CaM, PGK and protein 3 in sensory neurons

In the *in vitro* translation experiments discussed above, changes in mRNAs could have been caused by changes in levels of mRNAs or the "translatability" of mRNAs. Therefore, to begin investigating mechanisms for the change and to directly confirm the changes in mRNAs that were observed in the *in vitro* translation experiments, we developed ribonuclease protection assays (RPAs) to measure levels of mRNAs. RPAs are sensitive assays for quantifying levels of mRNAs. This increase in sensitivity has allowed us to investigate levels of mRNAs in clusters of sensory neurons obtained from 4 pleural ganglia. Another advantage of RPAs is that several mRNAs can be investigated in a given assay. Using the RPA technique on mRNA isolated from sensory neurons, we found that 1.5h 5-HT increased mRNA of CaM by 71% (N=7), protein 3 by 59% (N=7), and PGK by 33% (N=8) (Zwartjes, *et al.*, in preparation). These findings, which are all statistically significant, confirm our earlier results on these mRNAs (using *in vitro* translation) and indicate that 5-HT indeed affected levels of these mRNA in sensory neurons.

4. Effects of behavioral training on mRNAs

The effects observed previously on mRNAs were obtained using applications of 5-HT to isolated portions of the nervous system. An important issue is whether such changes occur during training of intact animals and the formation of memory. We have begun to address this issue by applying sensitizing stimuli over a 1.5 h period to one side of an *Aplysia*. After stimulation, levels of mRNA were measured in clusters of sensory cells from the stimulated side and compared to control levels of mRNA in sensory neurons from the unstimulated side. Behavioral training increased the level of protein 3 mRNA by 41% (N=5). Similar experiments are currently underway to investigate the effects of behavioral training on CaM and PGK.

5. 5-HT affects the level of an mRNA whose gene is very similar to the dorsal-ventral patterning gene (*tolloid*-gene) of *Drosophila*

A new technique called differential display reverse transcription-PCR (ddRT-PCR) allows for the investigation of changes in specific (but unidentified) mRNAs. Conceptually, this technique for studying mRNAs is similar to the use of 2D-gels to investigate specific proteins. Of 4 mRNAs that appeared to be affected, we first focused our attention on band 2 mRNA (Liu, *et al.*, 1995). To investigate if the difference observed using ddRT-PCR was reproducible, we used the RPA technique with riboprobes made to the band 2 mRNA. To do this, band 2 mRNA was eluted from the ddRT-PCR gel, amplified by PCR, cloned, and sequenced. 5-HT produced a significant increase in clone 2 mRNA (82%, N=10) obtained from clusters of sensory neurons.

After establishing that the effect of 5-HT on clone 2 mRNA was reproducible, and that it occurred in sensory neurons, we determined what was coded by the mRNA of clone 2. The gene for

clone 2 was obtained by screening on *Aplysia* head ganglia cDNA library with the cloned PCR fragment originally derived from the ddRT-PCR gel. Eight separate positive clones were isolated with different insert sizes (1.5-4.0Kb), and these have yielded the complete sequence of the gene. The sequence obtained was very similar to a family of genes containing the *Drosophila tolloid* gene and the gene coding for bone morphogenetic protein 1 of vertebrates (BMP-1). The overall identity of the *Aplysia* gene with members of the *tolloid*-BMP-1 family is 40-50%. This family of genes codes for a metalloendoprotease that is probably secreted and is believed to interact with (activate) members of the TGF-β family of growth factors (the *dpp* gene in the case of *Drosophila* and bone morphogenetic proteins 2-5 in the case of BMP-1) and/or to process procollagen.

Our finding that *Aplysia* "clone 2" is very similar to *tolloid* and BMP-1 and that "clone 2" is regulated by 5-HT is extremely interesting and very important. A large number of studies have shown that changes in morphology, growth, and formation of new synapses accompanies the formation of memory in *Aplysia* and in other organisms as well. An *Aplysia tolloid*-like protein may be involved in regulating some of the morphological changes that have been associated with long-term sensitization.

At this time, our working hypothesis is that the *Aplysia tolloid*-like protein is secreted and activated as a consequence of induction stimuli. Acting as a protease, Aptlb-1 then interacts with and activates another protein factor (perhaps an *Aplysia* TGF-β-like protein or procollagen). The function of Aptlb-1 could be to induce morphological or other changes that then would cause "long" term or "very-long" term synaptic facilitation. Since morphological changes appear rather quickly (right after 1.5h serotonin), Aptlb-1 may not be a primary mediator of the morphological changes. It is more likely that *tolloid* is involved in maintaining the changes that initially are put into motion by 5-HT. Therefore, Aptlb-1 could be part of a feedback control pathway that sustains the early memory. Such feedback factors have been proposed to play a role in differentiation (since it requires continuous active control) and in mechanisms for memory. This hypothesis concerning the action of *Aplysia tolloid* leads to many testable predictions.

We have investigated many other aspects of Aptlb-1. As the effects of 5-HT on facilitation are mediated by cAMP, we investigated whether increases in cAMP regulated Aptlb-1 mRNA in sensory neurons. Analogs of cAMP and forskolin? both produced significant increases in mRNA of Aptlb-1. Also, behavioral training had a significant effect on the mRNA of Aptlb-1. Another aspect we have begun to investigate is the protein product of Aptlb-1 mRNA. An antibody has been made to a protein expressed from Aptlb-1 mRNA. This antibody specifically recognized 2 proteins of the predicted molecular weights on Western Blots of proteins obtained from *Aplysia* ganglia. (The cDNA coding for Aptlb-1 has two initiation sites indicating that a cytosolic as well as secreted form of the protein may exist.) These experiments have set the stage for us to study the regulation and function of a protein in learning and memory. It is particularly interesting that, heretofore, Aptlb-1 has only been implicated in a developmental process.

B. Identification of a protein whose synthesis was affected by an analog of associative learning

A form of associative plasticity, activity-dependent neuromodulation, involves the convergence of neural activity and the effects of a modulatory neurotransmitter. This associative plasticity, which results in an enhancement of synaptic transmission, has recently been shown to have a long–term form lasting 24h as well as a short–term form lasting minutes. The existence of the long–term form raised the possibility that protein synthesis might be involved in this form of plasticity. Previously, the role of protein synthesis in associative plasticities has received very little attention. We examined if an analog of activity–dependent neuromodulation induced changes in protein synthesis in cells of *Aplysia* abdominal ganglia (Noel, *et al.*, 1994). The analog consisted of exposure of abdominal ganglia to paired treatments of elevated potassium (high K^+) to mimic spike activity and 5–HT to mimic the effects of sensory stimulation. We found one protein (P9) whose incorporation of label was significantly increased by the paired procedure but not by high K^+ or 5–HT applied alone. We first obtained two non–overlapping sequences of 36 and 37 amino acids from protein 9 using the microsequencing techniques. To align the peptides and to obtain additional sequence information, degenerate primers with Inosines at the points of highest degeneracy were synthesized to the 2 peptides and used to amplify cDNA synthesized from poly (A^+) mRNA from *Aplysia* ganglia. A band of approximately 180 bases was obtained, and it yielded an additional sequence of 19 amino acids between the 2 peptides. Therefore, a combined sequence of 92 amino acids was obtained from protein 9. This long peptide was found to have a 34% identity over an 88 amino acid region to the stringent starvation protein (SSP) of *E. coli*, a 43% identity over a 59 amino acid region to a Glutathione–S–transferase (GST) protein from *D. caryophyllus*, and a 40% identity over a 46 amino acid region of an auxin–induced protein (AIP) from *N. tabacum*. The similarities between protein 9 and each of SSP, GST and AIP are highly significant because the probabilities of obtaining such matches by chance are extremely low (3.7×10^{-16}, 2.0×10^{-6} and 6.5×10^{-7}, respectively). In addition, the SSPs and GSTs are related. An auxin–induced protein from *N. plumbaginifolia*, very similar to the AIP mentioned above, also has significant similarity to the SSP and GST families of proteins. The results of these experiments provide a basis for future investigations of the mechanisms by which associative forms of stimulation produce molecular effects.

C. Investigation of protein synthesis in *Halobacteria*

The recent discovery that a prokaryote, cyanobacteria, contains a circadian clock raised the possibility that other prokaryotes might also have clocks available to study. We chose to investigate *Halobacteria* because is was in a different kingdom from cyanobacteria, and it potentially has advantages, e.g., it is not photosynthetic.

The first question investigated was "does light affect protein synthesis?" To answer this, experiments were performed in which cell cultures, started from a single colony, were grown to various growth phases (early log, log, late log, and stationary) and were then checked for changes in

protein synthesis by radioactively labeling newly-synthesized proteins with 35S-methionine. Initial experiments were performed by growing cell cultures in both constant darkness and in 12-hour light/dark cycles, adding label to the culture media, exposing the cultures to 3-hour light pulses, processing the cell proteins, and running samples on 2-D polyacrylamide gels (2-D PAGE). No proteins were found whose synthesis was significantly changed by light.

The next question investigated was "is there a circadian rhythm in overall protein synthesis?" We performed experiments in which cell samples were grown in both 12:12 L/D and in constant D/D and were labeled with 35S-methionine every three hours over a 48-hour period. Total incorporated counts were measured by TCA precipitable counts. The results showed that while there was no conclusive evidence of circadian cycling in protein synthesis, there was some interesting parallel increases/decreases in protein synthesis between cultures grown in both L/D and D/D. To further pursue this, we ran 1-D PAGE gels of these protein samples. When the protein samples from both the L/D and D/D cultures were run on 2-D PAGE, visual analysis showed that the day (subjective day) time samples were noticeably darker than the night (subjective night) time samples. To ensure that temperature changes were not occurring with the light/dark cycles and subsequently causing changes in protein synthesis, we fabricated constant temperature environmental chambers in which to grow our cell cultures.

In the next series of experiments, we once again investigated the rhythms of synthesis but used both 2-D and 1-D PAGE of entrained cell samples grown to late log and to stationary growth phases under both L/D and D/D conditions. Samples were taken over a 36-hour period, labeled with 35S-methionine, processed and run on both gel formats. The resulting autoradiograms from these gels showed no significant changes in the synthesis of any specific proteins.

Therefore, the initial investigations of *Halobacteria* have been disappointing. To date, we have been unable to find proteins whose synthesis is regulated by light and we have been unable to find any evidence for a circadian clock. Our next approach will be to look for rhythms at the molecular level, i.e., rhythmic gene expression.

D. Regulation of proteins and genes that appear to be involved in the circadian timing system

1. Screening for protein components of circadian system:

A major problem in circadian biology is to identify the molecular components of the circadian oscillator. So far, very few candidates for such components have emerged. The best candidates, the products of the *per* gene and the *frq* gene, have come from mutational analysis of *Drosophila* and *Neurospora* strains, respectively. Such approaches, however, may not identify all clock components, and ultimately biochemical approaches also may need to be utilized to identify additional molecular components. We have used a biochemical approach to screen for proteins that may serve as components of the circadian oscillator in the eye of *Aplysia* (Koumenis and Eskin, 1992).

Our screening strategy is based on a model of the circadian system that has emerged from studies of *Drosophila* and *Neurospora* and is consistent with a considerable amount of data from the *Aplysia* eye. The model proposes that a clock gene is transcribed into mRNA and, soon thereafter, it is translated into protein. The oscillator feedback loop is completed when the protein directly, or through an effect on additional proteins, feeds back and affects it own expression as the level transcription, and perhaps, translation as well. This model predicts several properties of proteins involved in the circadian system: the synthesis of these proteins should be regulated by light, 5-HT, and brief treatments of the reversible transcription inhibitor DRB. Hence, we used these three treatments to screen for proteins (putative oscillator proteins, POPs) that may be components of the circadian oscillator. Additional properties of POPs are that perturbation of elements along light/5-HT entrainment pathways should change the synthesis of these proteins in the same manner as light/5-HT. For example, elevating intracellular cAMP and 5-HT should have similar effects on these proteins since cAMP is a second messenger in the 5-HT entrainment pathway. In essence, this "screen" for circadian system proteins attempts to correlate effects of treatments on the rhythm with the effects of the same treatments on the synthesis of proteins.

This "screen" for proteins has been an ongoing project. So far, we have used light (and analogs of cGMP) (Raju, *et al.* 1990), 5-HT (and analogs of cAMP) (Koumenis, *et al.* 1995), and DRB (Koumenis, *et al.* 1996) to search for proteins. Isolated eyes maintained in constant darkness were exposed to light, 5-HT or DRB. In some experiments, eyes were exposed to labelled amino acid during treatments and in other experiments eyes were exposed to labelled amino acid at various times after the treatment. This type of "screen" is "tricky" (as all "screens" are) because there are an endless number of ways or parameters to use (intensities, durations, phases, time after the experimental treatment) to look for proteins. Therefore, observing effects produced by a given treatment is much more important than observing no effects in a particular experiment (because we might not have used the right set of variables in our search).

2. Identification of proteins whose synthesis was perturbed by 1.5h, 5-HT or DRB

A variety of techniques including protein microsequencing, PCR, and cDNA cloning have been used to identify proteins. So far, we have obtained information on the identity of 5 proteins (Koumenis, *et al.*, 1995).

(a) 31K, 5.6 pI protein. The 31K protein originally was of interest because both light and DRB affected its synthesis. Two non-overlapping sequences of 37 amino acids and 36 amino acids were obtained from the 31K protein. Using degenerate primers made to regions of each peptide, cDNA from *Aplysia* ganglia, and the PCR technique, we obtained a band of 180 nucleotide bases and then cloned it. The sequence of this clone allowed us to fill in the amino acid sequence between the 2 peptides. Therefore, combining the two techniques of microsequencing and PCR, we obtained a sequence of 92 amino acids for the 31K protein (representing about one-third of the protein). This protein has significant similarity to a family of proteins containing auxin-induced protein (AIP) in

plants, stringent starvation protein (SSP) in *E. coli*, and Glutahione S-transferase (GST) from plants. AIPs and SSPs may act as transcript factors whereas GSTs are involved in cell protective mechanisms. The precise role of the 31K protein in *Aplysia* remains to be determined.

(b) 36K, 5.7 pI protein (porin). This protein was affected by light, 5-HT, and DRB. Using microsequencing techniques, two non-overlapping peptides of 30 and 25 amino acids were obtained. Each of these peptides was around 70% identical to human porin. Porins (also called VDACs for voltage-dependent anionic channels) are large conductance channel proteins which mainly conduct anions and are found in outer membranes of bacteria and mitochondri. Some evidence suggests that eukaryotic plasma membranes may contain porins as well. Using a full-length clone of human porin, the porin gene was obtained from a neuronal cDNA *Aplysia* library (Koumenis, *et al.* 1994). We now have the complete sequence of the *Aplysia* porin gene which is about 70% identical to the human porin gene.

(c) 40K, 5.6 pI protein (annexin). This protein was affected by light and DRB but so far it has not been found to be affected by 5-HT. A 38 amino acid sequence obtained from this protein was found to be more than 60% identical to sequences of a family of proteins called annexins. Furthermore, antibodies raised against annexin proteins reacted with the 40K *Aplysia* protein (Raju, *et al.* 1994). Annexins are a widely-expressed family of Ca^{++}-phospholipid binding proteins. Although they are currently under intensive investigation, specific roles for annexins have not been established. Proposed functions of annexins include membrane fusion (endocytosis and exocytosis), Ca^{++} channels, and regulation of arachidonic acid metabolism.

(d) 70K, 5.4 pI protein (HSP70). This protein has a great number of interesting properties. So far, it has been found to be regulated by light, 5-HT, analogs of cAMP, and DRB. The low amount of 70K protein normally present in eyes led us to use means other than microsequencing to identify the 70K protein. The 70K protein appears to be a member of the HSP70 (heat shock protein) family of proteins. The synthesis of this protein was dramatically increased by heat shock treatments to the eye (Koumenis, *et al.* 1995). In addition, treatment of eyes with $CdCl_2$, another treatment known to increase the synthesis of HSP70 proteins in other systems, also increased HSP70 in *Aplysia* eyes. HSP70 is found in normal conditions in the cytosol and at other times in the nucleus. It functions as a molecular chaperone in the passage of proteins from cytosol to the nucleus. Hence, the 70K protein could in some way be functionally homologous to the newly-discovered *timeless* gene that affects the *Drosophila* clock and the appearance of *per* protein in the nucleus (Sehgal, *et al.* 1994). It is also interesting that light regulates levels of HSP70 in algae and that correlations exist between effects of elevations of temperature on circadian rhythms and on expression of HSP70 in *Neurospora* (Gromoff, *et al.* 1989; Rensing, *et al.* 1987). Clones of the *Aplysia* HSP70 gene are available in our laboratory as a gift from Kuhl, *et al.* (Kuhl, *et al.* 1992). With this clone, we can study levels of HSP70 mRNAs.

(e) 78K, 5.1 pI protein (BiP). This protein also has a number of interesting properties. Its synthesis was found to be regulated by light, 5-HT, and DRB. Two non-overlapping sequences of 40 amino acids were obtained from this protein. Each of these sequences was more than 90% identical to

sequences of human binding protein (BiP)/Glucose Regulated Protein 78 (GRP). This BiP protein in the eye is similar to one studied by Kuhl, *et al.* in *Aplysia* ganglia. Kuhl, *et al.* found that 5-HT regulated the synthesis of this protein and the expression of the BiP gene (Kuhl, *et al.* 1992). BiP resides in the lumen of the endoplasmic reticulum and also functions as a molecular chaperone. Its main function is to assist in the correct folding of proteins as well as in the current assembly of oligomeric proteins into complexes.

3. Role of transcription in rhythm regulation and generation

(a) Inhibition of transcription with DRB. In our first experiments using inhibitors of transcription published in Science (Raju, *et al.* 1991), we showed that brief treatments of DRB (2h) inhibited transcription and phase shifted the rhythm, whereas continuous treatments altered the period of the rhythm. These were the first experiments that correlated the effects of an inhibitor of transcription with its effects on the circadian rhythm. Subsequently, others have used DRB in a number of systems and have obtained results similar to ours (e.g., Ohi, *et al.* 1991; Liu, *et al.* 1992). Recently, more direct evidence for a role of transcription in the circadian oscillating mechanism has emerged from experiments on *Drosophila* and *Neurospora* (Aronson, *et al.* 1994; Hardin, *et al.* 1990). Over the past year and a half, we further studied transcription by investigating several issues (Koumenis, *et al.* 1996). (**1**) What is the effective time course of inhibition of transcription for brief DRB treatments, i.e., how fast does transcription return to normal levels after removal of DRB? The effective period of inhibition for a 0.5h DRB treatment is 1.5h (0.5h phase plus 1h recovery). (**2**) Do 0.5h treatments of DRB phase shift the rhythm? Phase shifts in the rhythm as large as 2.5h were produced by 0.5h DRB treatments. Therefore, inhibition of transcription for 1.5h can phase shift the rhythm. This indicates that mRNAs with relatively brief half-lives are involved in the oscillator mechanism. (**3**) What is the phase of sensitivity of the rhythm to DRB? We obtained a complete phase response curve using 0.5h treatments of DRB. The phase of maximum sensitivity to DRB was found to be CT4-6 and the period of sensitivity ranged from CT0 to CT14. (**4**) How soon after DRB treatments are phase shifts apparent? How closely coupled are transcription, translation, and the circadian output system? When DRB was given CT3:00-3:30, the phase shift in the rhythm was apparent within 2h after the end of the treatment. Thus, the rhythm appears to be phase shifted as soon as transcription returns to its normal level following treatment with DRB. This rapid appearance of the phase shift indicates that a protein with a brief half-life (~2h) is also involved in the oscillator mechanism and that transcription, translation, and the output pathways are tightly coupled together. (**5**) Is the synthesis of specific proteins inhibited during and/or shortly after a DRB treatment as predicted above? DRB produced no general, overall effects on protein synthesis but it did affect the synthesis of a few specific proteins. We found that the synthesis of 2 proteins was increased during 2h DRB treatments and the synthesis of 15 proteins was affected (10 increased, 5 decreased) during the 2h period after 2h DRB treatments. Therefore, brief treatments of DRB do affect the synthesis of some proteins and the translation of some proteins

is affected rather quickly, i.e., within the time frame in which the rhythm appears to be phase shifted. In addition, several of the proteins whose synthesis was affected by DRB were proteins we previously found affected by light and/or 5-HT. This finding strengthens the hypothesis that these proteins play roles in the circadian system.

(b) Regulation of levels of mRNAs by light and 5-HT. A very important issue is whether the mRNAs of the putative oscillator proteins are regulated by light and 5-HT. As the model of the circadian system predicts, light/5-HT entrains the clock by either regulating mRNAs (transcription) or protein synthesis (translation). We already know that light and/or 5-HT regulate the synthesis of the 7 POPs. If light/5-HT is found to regulate levels of mRNAs, then this supports the hypothesis that light/5-HT acts at the transcriptional level and it would strengthen the involvement of the proteins in the circadian system.

Levels of mRNA for porin were altered significantly by light and 5-HT. This is interesting, as it is the first time that an external stimulus has been shown to regulate porin at both the protein and gene level in any organism. 5-HT treatments increased the levels of mRNA and protein of both BiP and porin. Also, light decreased the levels of the mRNA and protein of porin. These results suggest that light and 5-HT may act at the transcriptional level and that the transcription and translation of these proteins are closely coupled. Light treatments decreased the mRNA level of BiP and increased the protein level. This bi-directional affect suggests that light regulates the transcription and translation of BiP by different mechanisms than how it regulates porin. Perhaps light first increases BiP protein level which then leads to a decrease in BiP mRNA. These findings suggest that BiP and porin may be involved in the eye circadian system, and that the action of light and 5-HT on the circadian oscillator could occur by regulating levels of BiP and porin mRNAs and proteins. Whether they are *bona fide* oscillator components, input/output pathway components, or something else is unknown.

E. Inhibition of specific gene expression

A critical way to examine the role of a gene/protein in circadian rhythms (or any process for that matter) is to determine the effects on rhythms of directly varying the expression of that gene and level of its protein. Technology to do this using antisense oligodeoxynucleotides (ODNs) is developing rapidly. Eventually, we wish to use these techniques to eliminate or alter the levels of POPs. There are two methods to do this. One way is to inhibit the induction of the gene by interfering with activation of its promoter. Another way is to use antisense ODNs to bind a complementary mRNA sequence and prevent translation of the mRNA. To develop these techniques, we focused on the first method because of the availability of ODNs. Our strategy to develop the techniques in *Aplysia* is to use the heat shock induction of HSP70 in abdominal ganglia, and then once established, investigate whether the techniques work on the eye. A graduate student who recently graduated (Costas Koumenis) spent considerable effort on this project with some success.

Experimental abdominal ganglia were preincubated with a solution of double-stranded phosphorothioate ODNs, that contained the Heat-Shock Element (HSE). The HSE is a DNA element found upstream of all heat shock-induced genes, and it is the site of binding of the Heat Shock Factor protein upon heat shock. Replacing some of the oxygen atoms in ODNs with sulfurs are thought to make ODNs more resistant to degradation and perhaps more permeable to cell membranes. After 12 hours of preincubation with ds-ODNs containing the HSE, experimental eyes were exposed to ^{3}H-leucine and heat shocked (lane 3 results) whereas one set of control eyes was heat shocked and exposed to label (lane 2 results). Another set of control eyes was only exposed to ^{3}H-leucine and not heat shocked. Heat shock induced a 70K protein and other smaller molecular weight ones (compare lanes 1 and 2). The ds-ODNs blocked induction of the 70K protein. Presumably this blockage of induction occurred because the extra HSE elements added to the cell bound the Heat Shock Factor proteins and thus prevented them from inducing HSP70 protein. This experiment was repeated two more times and gave similar results. These are very encouraging results indicating that we may be able to use this type of approach to manipulate genes/proteins in the isolated eye.

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A. Papers published

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Zwartjes, R.E., Noel, F., Nuñez-Regueiro, M., Homayouni, R., Cook, R., Crow, M.J., Byrne, J.H. and Eskin, A. 1996. Treatments producing long-term facilitation affect specific mRNAs and proteins in Aplysia neurons (submitted).

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Koumenis, C., Nuñez-Regueiro, M. and Eskin, A. 1992. Identification of three additional putative oscillator proteins (POPs) from the eye of Aplysia as stress-related proteins. Soc. Neurosci. Abst. 18: 881.

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V. Personnel supported by grant:

Name	Title	[redacted]	Dates	% Effort
1. Benedik, Michael	Assoc. Professor		7.1.92 > 7.31.92 6.1.93 > 6.30.93	.50 1.00
2. Byrne, Susan	Lab Assistant		9.1.94 > 9.20.94	.50
3. Chen, Haixu	Lab Assistant		9.4.92 > 12.31.92	.50

V. Personnel supported by grant: (continued)

Name	Title	Dates	% Effort
4. Chen, Zhong	Lab Assistant	7.1.92 > 12.31.92	.50
5. Cheung, Hiu C.	Lab Assistant	7.1.92 > 9.30.92	.50
6. Darhower, Adrienne	Lab Assistant	3.9.95 > 8.31.95	.50
7. Hattar, Samer	Graduate Student	6.1.94 > 5.31.95	.50
8. Homayouni, Ramin	Graduate Student	7.1.92 > 12.31.93 1.1.95 > 8.31.95	.50
9. Koumenis, Constantinos	Graduate Student	7.1.92 > 8.31.92 12.1.92 > 5.31.93	.50
10. Lee, Oi Y.	Lab Assistant	12.8.92 > 12.29.92	.50
11. Liu, Jin	Lab Assistant	5.27.93 > 12.10.93	.50
12. Liu, Qing-Rong	Research Asst. Prof.	11.1.93 > 8.31.95	.50
13. Mills, Charles	Lab Assistant	5.27.94 > 5.31.95	.50
14. Nuñez-Regueiro, Marta	Lab Supervisor	7.1.92 > 9.30.92	1.00
15. Phifer, James	Lab Assistant	11.24.92 > 3.9.93	.50
16. Quigley, Kathleen	Research Assoc. Prof.	9.1.93 > 8.31.94	.50
17. Sloan, Mark	Graduate Student	9.1.92 > 5.31.94 7.1.94 > 5.31.95	.50
18. Urizar, Nancy	Lab Assistant	9.1.94 > 8.24.95	.50
19. West, Henry	Graduate Student	1.1.95 > 8.31.95	.50
20. Zwartjes, Raymond	Graduate Student	7.1.92 > 12.31.92	.50

3.21.96